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| Application of: | Liew, C.C. | Examiner: | Juliet C. Switzer |
| Serial No.: | 10/268,730 | | |
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| Titled: | Method for the Detection of Gene Transcripts in Blood and Uses Thereof | Conf. No.: | 5174 |

CERTIFICATE OF MAILING UNDER 37 C.F.R. § 1.8a

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***Declaration under U.S.C. 1.132***

I, Choong-Chin (C.C.) Liew, a citizen of 81 Millersgrove Drive, Toronto, Ontario, Canada M2R 3S1, declare the following:

That I am the inventor on the instant application.

That I have read and reviewed the 112 first paragraph enablement rejection applied in the Office Action dated January 4, 2006. I understand that the enablement rejection was further discussed in an interview at the USPTO with the Examiner and Applicant's representatives on February 7, 2006. This declaration is designed to address issues raised by the examiner regarding the enablement of the invention.

That I understand one issue prompting the enablement rejections is that the Examiner questions the broad application of the Applicant's invention. I also understand that the Examiner questions the unpredictability of applying the Applicant's invention given the sample size of the disclosed working examples and the lack of a demonstrated statistically significant relationship between the disease phenotype and the differential expression of the disclosed genes.

That I am using this declaration as an opportunity to extend the working examples of the

specification which provide for methods of detecting a difference in expression of a gene in an unfractionated sample of whole blood by detecting a difference in the amount of RNA from a human test subject versus a human control subject, wherein said difference is indicative of disease. Included herein are data showing the detection of differentially expressed genes using these claimed methods as applied to each of eleven unique diseases.

That the eleven diseases covered in this declaration include a wide range of diseases including infectious disease (Chagas disease), cardiac disease (Heart Failure, Chagas, Coronary Artery Disease), neurological disease (Alzheimer's disease, Schizophrenia), musculoskeletal disease (Osteoarthritis, Rheumatoid arthritis) as well as various cancers from diverse tissues (Liver Cancer, Prostate Cancer, Ovarian Cancer, Colorectal Cancer).

That for each of these diseases, statistical analysis is provided to show that there are numerous genes each of which demonstrate a statistically significant relationship of the level of RNA detected in an unfractionated sample of whole blood of patients with a specific disease as compared with controls without said disease, and with the differential expression being indicative of the disease under study. The sample sizes of these data sets presented herein are much larger than those disclosed in the specification, with the number of blood samples tested per disease being as high as sixty, and the number of differentially expressed genes analyzed per disease being as high as 3,925.

That the steps used to generate the data consisted of the following

1. Ten mls of peripheral whole blood were collected in Vacutainer tubes containing EDTA (Becton Dickinson, Franklin Lakes, N.J.). Patients and controls were recruited from various Institutions and each patient was diagnosed by a registered physician.

2. Total RNA was isolated from unfractionated whole blood by first treating the blood with lysis buffer (1.6 mM EDTA, 10 mM $KHCO_3$, 153 mM $NH_4Cl$, pH 7.4) and applying 1.0 ml of TRIzol® Reagent (Invitrogen Corp., Carlsbad, CA) and 0.2 ml of chloroform to the resulting pellet in accordance to the manufacture's instructions yielding approximately 20-30ug of RNA.

3. For each individual tested, five μg of the total RNA sample isolated was used for hybridization on a Affymetrix® U133Plus2 GeneChip (Affymetrix, Santa Clara, CA) following the manufacturer's instructions. The presence of each gene for a given sample was determined by the GeneChip Operating System (GCOS) software (Affymetrix, Santa Clara, CA) which provides a **detection p value** indicative of the likelihood that the gene was present or not. Probe sets identified as absent were not utilized for further analysis. RNA was hybridized with the Affymetrix® GeneChip in accordance with the provided protocol. Briefly RNA samples were reverse-transcribed to cDNA using an T7 oligodT primer. Following second strand synthesis, labeled RNA is synthesized using the Enzo® BioArray High Yield RNA labeling kit using biotinylated nucleotides. Resulting labeled RNA is fragmented prior to hybridization. To allow comparison amongst samples, signal intensities were normalized using the GeneSpring v.6.0 software and differentially expressed genes identified by applying known statistical tests (as specifically noted below for each example). Genes were identified which differentiated as between disease

and control samples at a set p value. Results were then visualized and agglomerative hierarchical cluster analysis performed using GeneSpring v 6.0 software.

4. For real-time RT-PCR assays, forward and reverse primers were designed using "PrimerQuest"by Integrated DNA Technologies, Coralville, IA. Oligo(dT)-primed 1st strand cDNA was synthesized with an Applied Biosystems High Capacity cDNA Archive Kit (cat # 4322171), on a Perkin-Elmer DNA Thermal Cycler, according to the manufacturer's protocol. Specific cDNAs were quantitated by real-time PCR, with the Qiagen Quantitect SYBR® Green PCR Kit (cat # 204143). Amplicons were detected, using either Opticon I DNA Engine (MJ Research) or ABI 7500 (ABI) with confirmation of specific amplification determined by calculated melting dissociation curve. Relative fold change of each individual gene was calculated using the comparative $\Delta Ct$= Ct (target gene) - Ct (house-keeping gene) where Ct values of target genes were normalized to housekeeping gene (β-actin).

That for each of 11 diseases discussed below, the data is presented in three parts (Part A, Part B, Part C).

That **Part A** is a dendrogram generated to visualize the microarray data generated using GeneSpring v6.0 which displays diagrammatically the differential expression levels of detected RNA as between disease and control samples for each of the genes identified. The dendogram is colour coded such that the color represents the intensity of the amount of RNA. Red represents genes that are more abundantly expressed, and blue represents genes that are less abundantly expressed. Each column in the dendogram identifies the results from a single individual and each row represents the results of a single gene as determined using one or more oligo probe sets corresponding to that gene. Agglomerative hierarchical clustering is noted above each dendogram as a tree diagram and represents the similarity as between the samples.

Conclusion: Each dendrogram displays results of a number of genes which were identified as differentially expressed as between disease and control at a statistically significant level which each demonstrated statistically significant differential expression as between individuals with disease patients from that of controls.

That **Part B** is an analysis determining the number of differentially expressed genes identified in each disease data set which were found to be unique to the disease under consideration as compared with the other diseases.

Conclusion: the majority of the differentially expressed genes are not part of a non-specific disease response.

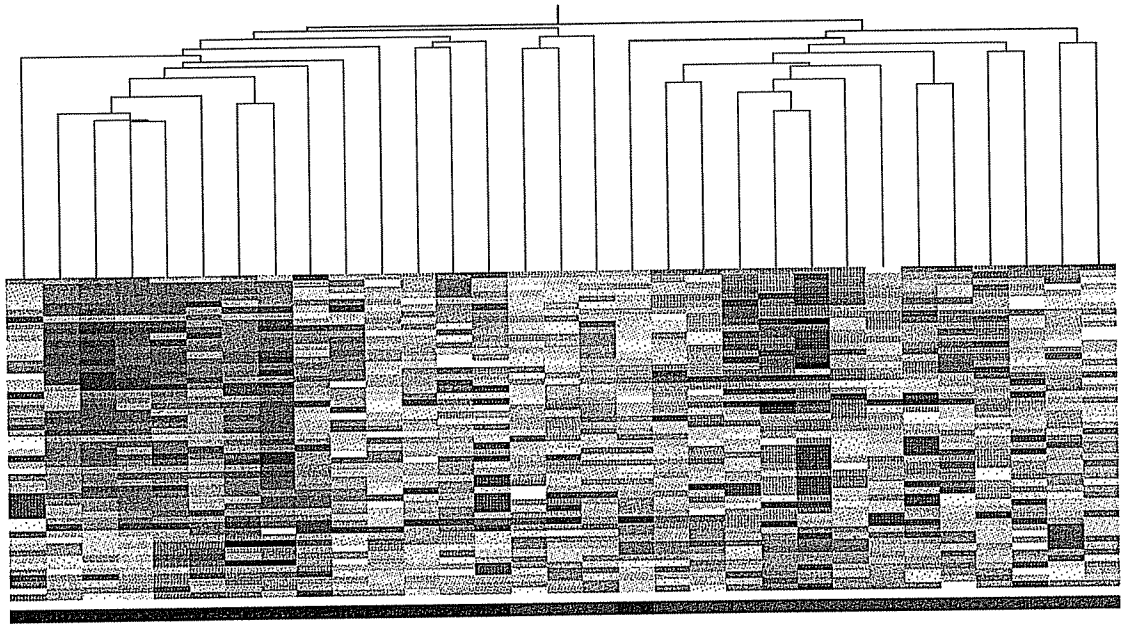
That **Part C** is a statistical analysis comparing the expression in diseased and control patients of each of a subset of the differentially expressed genes identified in part (A), as determined by Real-time RT-PCR.

Conclusion: The real time RT-PCR data demonstrates the use of a second and

independent method which validates the results of the microarray data.

**Example 1. Colorectal cancer** (CRC):

A. Microarray: a total of 31 samples from 15 non-CRC (controls) subjects and 16 CRC patients (where patients with CRC included individuals with various levels of colorectal cancer as determined in accordance with Duke staging criteria including 2 individuals stage A, 6 individuals stage B and 8 individuals with stage C colorectalcancer) were profiled using Affymetrix GeneChip U133 plus2.0, a microarray with probe sets corresponding to all known genes of the human genome. A set of 51 probe sets each corresponding to a gene ($p<0.001$, non-parametric, Wilcoxon-Mann-Whitney test) were identified which each demonstrated differential expression as between individuals with CRC patients from that of controls.



Control (n=15) CRC (n=16)

**p<0.0001**
**51 probes**

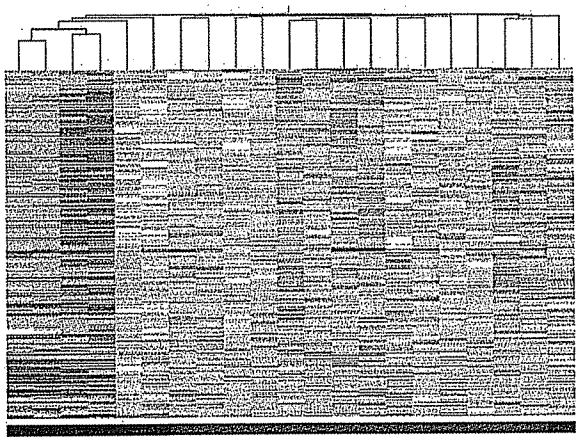
B. We have compared the 51 genes identified as noted above with the genes identified in each of the other lists described herein so as to demonstrate that many of the genes identified are indicative of colorectal cancer and not part of a general and non specific disease response. We concluded that 29 of the genes identified are unique to Colorectal Cancer and are not identified in the lists indicative of Alzheimer's, Bladder Cancer, Coronary Artery Disease (CAD), Chagas Disease, Heart Failure (HF), Liver Cancer, Osteoarthritis, Ovarian Cancer, Prostate Cancer, Rheumatoid Arthritis (RA) and Schizophrenia.

C. Real-time RT-PCR: Sixteen genes identified either by the microarray experiment described above, or other similar microarray experiments. These sixteen genes were tested using real time RT-PCR on a total of 115 samples (including 58 CRC samples and 57 controls). Thirteen genes were demonstrated to be significantly down-regulated as between patients having CRC as compared with the control patients, whereas three genes were demonstrated to be significantly up-regulated as between individuals having CRC when compared with control patients.

| Symbol | Microarray | | real-time RT-PCR CRC/Ctrl (58/57) | |
|---|---|---|---|---|
| | p value | CRC/Ctrl | p value | CRC/Ctrl |
| SPAP1 | 2.0E-03 | 0.74 | 0.00000001 | 0.40 |
| BCNP1 | 6E-05 | 0.61 | 0.00000000 | 0.42 |
| MS4A1 | 1.4E-02 | 0.61 | 0.00000000 | 0.42 |
| BANK1 | 3.6E-05 | 0.52 | 0.00000000 | 0.43 |
| OSBPL10 | 7.8E-09 | 0.51 | 0.00000004 | 0.45 |
| COBLL1 | 4.5E-05 | 0.66 | 0.00000054 | 0.47 |
| BLNK | 8E-07 | 0.56 | 0.00000000 | 0.51 |
| LTBP3 | 1.2E-04 | 0.43 | 0.00028011 | 0.55 |
| UTS2 | 2.4E-04 | 0.67 | 0.04243446 | 0.63 |
| LOC283130 | 5.1E-04 | 0.63 | 0.00000812 | 0.63 |
| KIAA1559 | 6.4E-03 | 0.69 | 0.00000027 | 0.64 |
| C9orf85 | 1.3E-03 | 0.67 | 0.00000427 | 0.65 |
| ANK3 | 1.4E-02 | 0.80 | 0.03458547 | 0.66 |
| MGC20553 | 6.2E-05 | 1.59 | 0.02322844 | 1.29 |
| GBP5 | 6.5E-03 | 1.57 | 0.03263835 | 1.32 |
| CDA | 1.5E-02 | 1.39 | 0.00009210 | 1.34 |

**Example 2: coronary artery disease (CAD)**

A. Microarray: A total of 21 samples (including 14 CAD and 7 controls) were profiled using the Affymetrix GeneChip U133 plus2.0. A set of 678 probe sets, each corresponding to a human gene were identified ($p<0.01$ Wilcoxon-Mann-Whitney non-parametric test) were identified which each demonstrated differential expression as between individuals with the CAD and the control patients. CAD patients were those diagnosed with single or multi-vessel CAD prior to angioplasty. Control subjects demonstrated no indication of cardiovascular disease.



Control (n=7) CAD (n=14)

**$p<0.01$**
**678 genes**

B. We have compared the 678 genes identified as noted above with the genes identified in each of the other lists noted so as to demonstrate that the probe sets identified can be considered indicative of colorectal cancer and not as part of a general and non specific disease response. We concluded that 258 of the genes identified are unique to CAD and are not identified in the lists indicative of Colorectal Cancer, Alzheimer's, Bladder Cancer, Chagas Disease, Heart Failure (HF), Liver Cancer, Osteoarthritis, Ovarian Cancer, Prostate Cancer, Rheumatoid Arthritis (RA) and Schizophrenia.

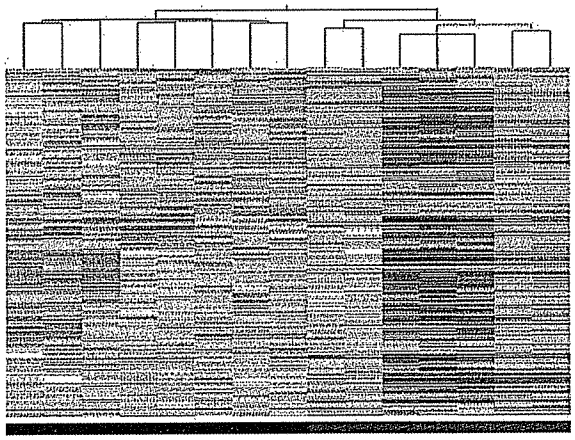
C. Real-time RT-PCR: the expression levels for 13 genes were assayed and 11 genes were verified as statistical significant ($p<0.05$) between control (n=14) and CAD (n=19) blood samples. The average fold change as between the CAD subjects and the control subjects is also noted.

| Gene | Gene Name | fold (CAD/Control)) | p Value |
|---|---|---|---|
| ABHD5 | Abhydrolase domain | 3.0075531 | 0.000303 |

| | | | |
|---|---|---|---|
| | containing 5 | | |
| CD3D | CD3D antigen, delta polypeptide | 0.4062744 | 0.001022 |
| CD96 | CD96 antigen | 0.3941159 | 0.003763 |
| CHS1 | lysosomal trafficking regulator | 0.5768841 | 0.04025 |
| CRTAM | class-I MHC-restricted T cell associated molecule | 0.5051862 | 0.024476 |
| HNRPH1 | heterogeneous nuclear ribonucleoprotein H1 | 0.503045 | 0.044293 |
| IGF2R | insulin-like growth factor 2 receptor | 2.0343497 | 0.001574 |
| MMP25 | matrix metallopeptidase 25 | 5.1250824 | 0.016486 |
| ABCA1 | ATP-binding cassette, sub-family A (ABC1), member 1 | 3.1116676 | 0.006021 |
| SEC14L1 | SEC14-like 1 (Alias SEC14L) | 3.2748921 | 0.00018 |
| TXNDC5 | thioredoxin domain containing 5 | 0.4784711 | 0.01799 |

**Example 3: Heart Failure (HF)**

A. Microarray: A total of 15 samples (including 7 HF and 8 controls) were profiled using the Affymetrix GeneChip U133 plus2.0. A set of 3,925 probe sets, each corresponding to a gene ($p<0.01$, Wilcoxon-Mann-Whitney non-parametric test) were identified which each demonstrated differential expression as between individuals with the HF and the control patients. Patients with HF were diagnosed with end-stage heart failure (median NYHA class 4), and controls were subjects without known heart failure.



**P<0.01**
**3,925 genes**

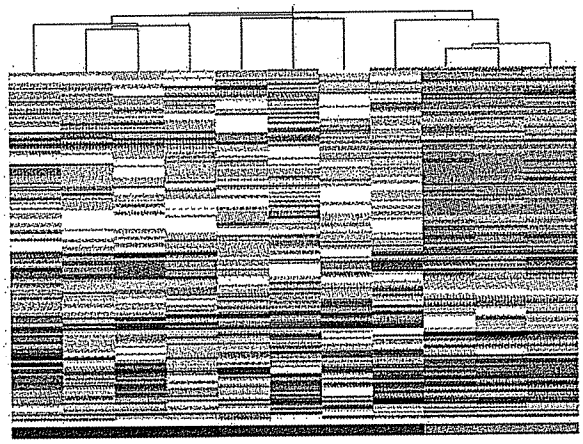
B. We have compared the 3,925 genes identified as noted above with the genes identified in each of the other lists described herein so as to demonstrate that the genes identified can be considered indicative of heart failure and not as part of a general and non specific disease response. We concluded that 1,925 genes are unique to HF and are not identified in the lists indicative of Colorectal Cancer, Alzheimer's, Bladder Cancer, Chagas Disease, Coronary Artery Disease (CAD), Liver Cancer, Osteoarthritis, Ovarian Cancer, Prostate Cancer, Rheumatoid Arthritis (RA) and Schizophrenia.

D. C. Real-time RT-PCR: The expression levels for 5 genes were tested and 4 genes were verified as statistical significant ($p<0.05$) between control ($n=8$) and end-stage heart failure ($n=11$) blood samples. The average fold change as between the CAD subjects and the control subjects is also noted.

| Gene | Gene Name | Fold Change HF v. Control | p Value |
|---|---|---|---|
| MYL4 | myosin, light polypeptide 4, alkali; atrial, embryonic | 0.012158297 | 0.004664623 |
| MAP2K3 | Mitogen-activated protein kinase kinase 3 | 0.115356341 | 0.00003 |
| RNF10 | ring finger protein 10 | 0.072452457 | 0.0000003 |

**Example 4: Chagas' heart disease**

A. Microarray: a total of 11 samples (including 3 symptomatic, 4 asymptomatic Chagas' heart disease and 4 controls) were profiled using Affymetrix GeneChip U133 plus2.0. A set of 155 probe sets each corresponding to a gene ($p<0.01$) were identified were identified which each demonstrated differential expression as between individuals with asymptomatic Chagas, symptomatic Chagas and individuals not having Chagas disease.



Control (n=4) Asymptomatic (n=4) Symptomatic (n=3)

**p<0.01**
**155 genes**

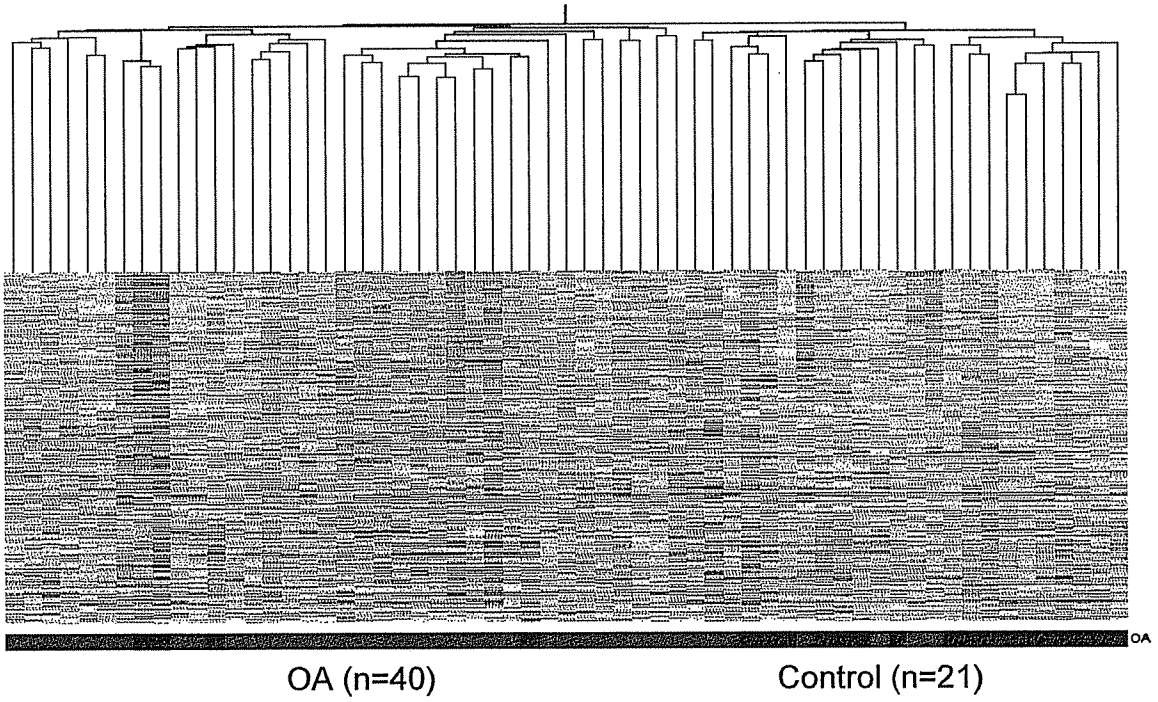
B. We have compared the 155 genes identified as noted above with the genes identified in each of the other lists described herein so as to demonstrate that the genes identified can be considered indicative of heart failure and not as part of a general and non specific disease response. We concluded that 79 of the genes identified are unique to Chagas Disease and are not identified in the lists indicative of Colorectal Cancer, Alzheimer's, Bladder Cancer, Heart Failure (HF), Coronary Artery Disease (CAD), Liver Cancer, Osteoarthritis, Ovarian Cancer, Prostate Cancer, Rheumatoid Arthritis (RA) and Schizophrenia.

C. Real-time RT-PCR: The expression levels for 4 genes were tested and all 4 genes were verified as statistical significant ($p<0.05$) between control (n=4) and Chagas' (n=5) samples.

| Gene | Gene Name | Fold Change Chagas v Control | P Value |
|---|---|---|---|
| ABCA1 | ATP-binding cassette, sub-family A (ABC1), member 1 | 3.625375674 | 0.020174 |
| CD69 | CD69 antigen (p60, early T-cell activation antigen) | 0.212821159 | 0.012406 |
| CDC14A | CDC14 cell division cycle 14 homolog A (S. cerevisiae) | 0.613811678 | 0.026988 |
| TXNIP | thioredoxin interacting protein | 0.357220926 | 0.007264 |

**Example 5: Osteoarthritis (OA)**

A. Microarray: a total of 61 samples (including 40 OA and 21 non-OA or controls) were profiled using Affymetrix GeneChip U133 plus2.0. A set of 2172 probe sets, each corresponding to a gene ($p<0.05$, Wilcoxon-Mann-Whitney non-parametric test) were identified which each demonstrated differential expression as between individuals with Osteoarthritis and individuals without Osteoarthritis. Note individuals who appear to be incorrectly classified are noted below.



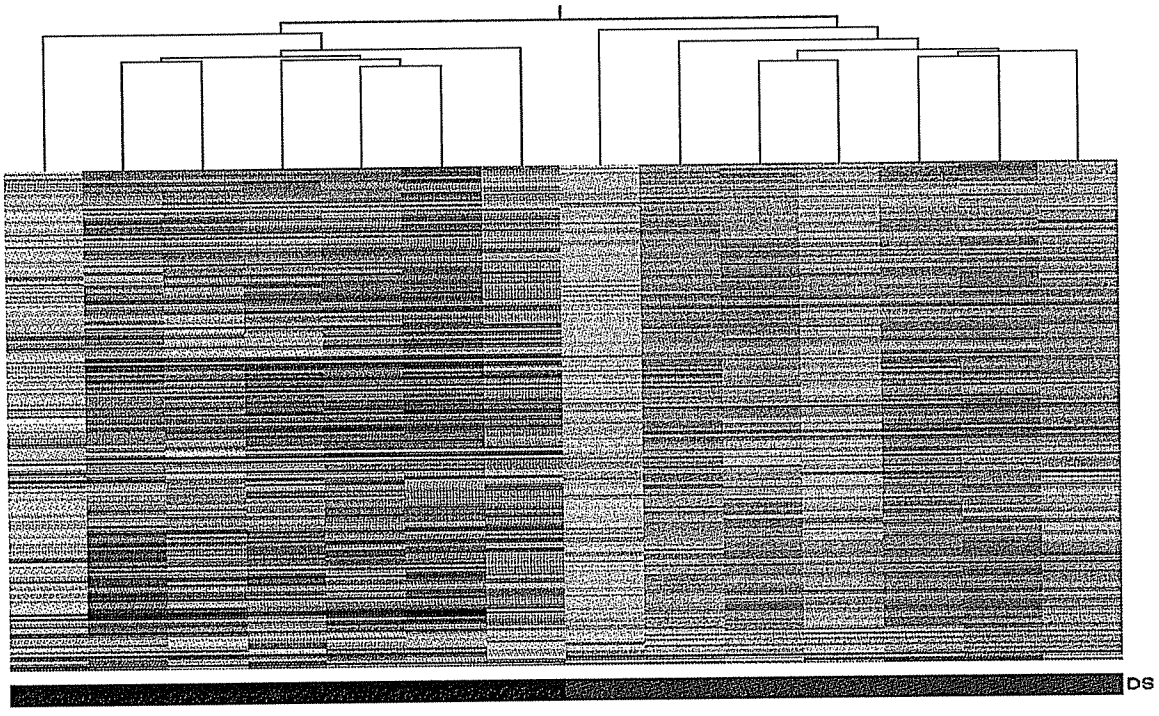

B. We have compared the 2171 genes identified as noted above with the genes identified in each of the other lists described herein so as to demonstrate that the genes identified can be considered indicative of osteoarthritis (OA) and not as part of a general and non specific disease response. We concluded that 1,590 of the genes identified are unique to OA and are not identified in the lists indicative of Colorectal Cancer, Alzheimer's, Bladder Cancer, Heart Failure (HF), Coronary Artery Disease (CAD), Liver Cancer, Chagas Disease, Ovarian Cancer, Prostate Cancer, Rheumatoid Arthritis (RA) and Schizophrenia.

C. Real-time RT-PCR: The expression levels for 9 genes were tested and 6 genes were verified as statistical significant ($p<0.05$) between control (n=49) and OA (n=196) samples.

| | OA/Control | |
|---|---|---|
| | p-value | Fold Change |
| ATP1B1 | 0.001 | 1.33 |
| CPT1A | 5.48E-09 | 1.52 |
| LRMP | 0.011 | 0.86 |
| PDK4 | 1.36E-05 | 1.45 |
| F2RL1 | 0.016 | 0.75 |
| IL13RA1 | 1.23E-10 | 0.69 |

**Example 6: Alzheimer's Disease (AD)**

A. Microarray: A total of 14 samples (including 7 AD patients and 7 controls) were profiled using Affymetrix GeneChip U133 plus2.0. A set of 200 probe sets, each corresponding to a gene ($p<0.05$, Welch t-test Bonferroni $p<0.05$) were identified which each demonstrated differential expression as between individuals with alzheimer's disease and individuals without alzheimer's disease.




Control (n=7) AD (n=7)

**p<0.05**
**200 genes**

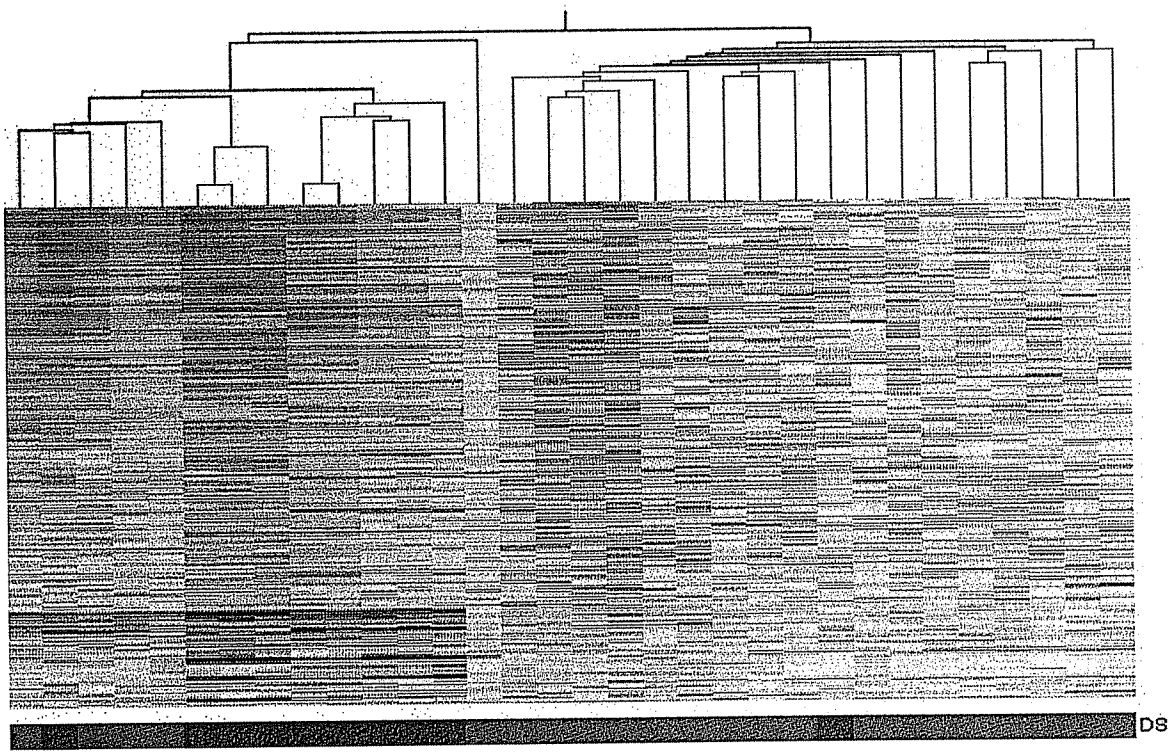
B. We have compared the 200 genes identified as noted above with the genes identified in each of the other lists described herein so as to demonstrate that the genes identified can be considered indicative of Alzheimer's and not as part of a general and non specific disease response. We concluded that 114 of the genes identified are unique to Alzheimer's and are not identified in the lists indicative of Colorectal Cancer, Osteoarthritis, Bladder Cancer, Heart Failure (HF), Coronary Artery Disease (CAD), Liver Cancer, Chagas Disease, Ovarian Cancer, Prostate Cancer, Rheumatoid Arthritis (RA), and Schizophrenia.

**Example 7: Schizophrenia**

A. Microarray: A total of 30 samples (including 20 schizophrenia subjects and 10 control subjects not having schizophrenia) were profiled using Affymetrix GeneChip U133 plus2.0. A set of 2064 probe sets, each corresponding to a gene ($p<0.005$, Wilcoxon-Mann-Whitney non-parametric test) were identified which each were differentially expressed as between individuals having schizophrenia and individuals not having schizophrenia.

B. We have compared the 200 genes identified as noted above with the genes identified in each of the other lists described herein so as to demonstrate that the genes identified can be considered indicative of Alzheimer's and not as part of a general and non specific disease response. We concluded that 116 of the genes identified are unique to Alzheimer's and are not identified in the lists indicative of Colorectal Cancer, Osteoarthritis, Bladder Cancer, Heart Failure (HF), Coronary Artery Disease (CAD), Liver Cancer, Chagas Disease, Ovarian Cancer, Prostate Cancer, Rheumatoid Arthritis (RA), and Schizophrenia.

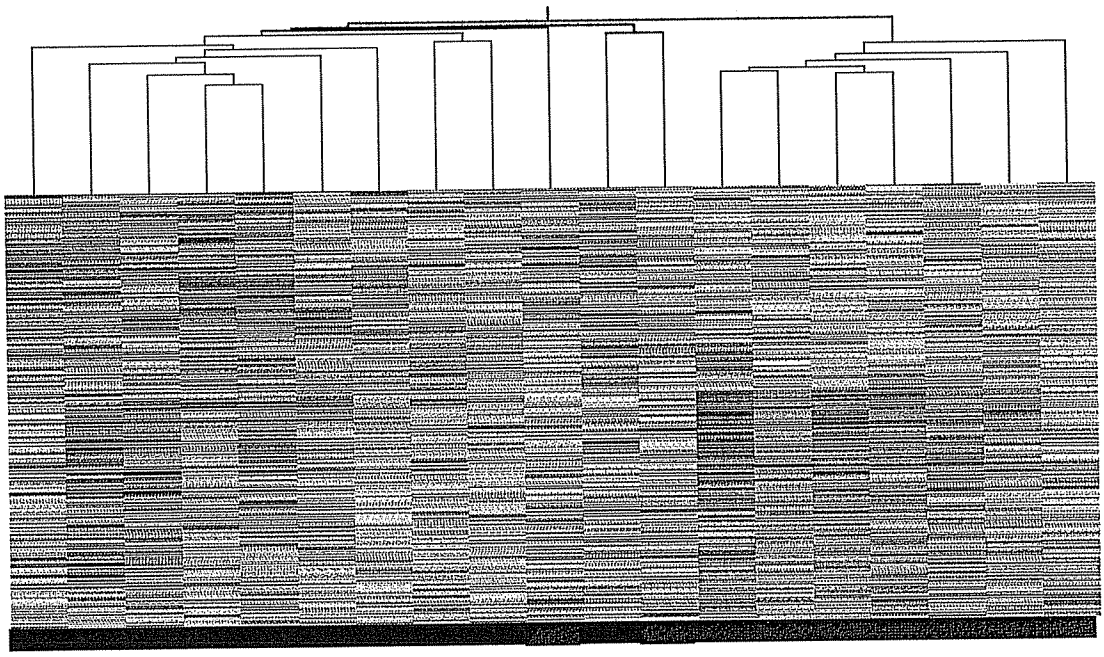
C. Real-time RT-PCR: The expression levels for 13 genes were tested and 8 genes were verified as demonstrating statistically significant differential expression ($p<0.05$) as between individuals not having schizophrenia ($n=14$) and individuals having schizophrenia ($n=15$ to 25).




p<0.005
2,064 genes

**Example 8: Rheumatoid Arthritis (RA)**

A. Microarray: A total of 19 samples (including 10 RA and 9 controls) were profiled using Affymetrix GeneChipU133 Plus2.0. A set of 463 probe sets, each corresponding to a gene ($p<0.01$, Wilcoxon-Mann-Whitney non-parametric test) were identified which each were differentially expressed as between individuals having rheumatoid arthritis and individuals not having rheumatoid arthritis.



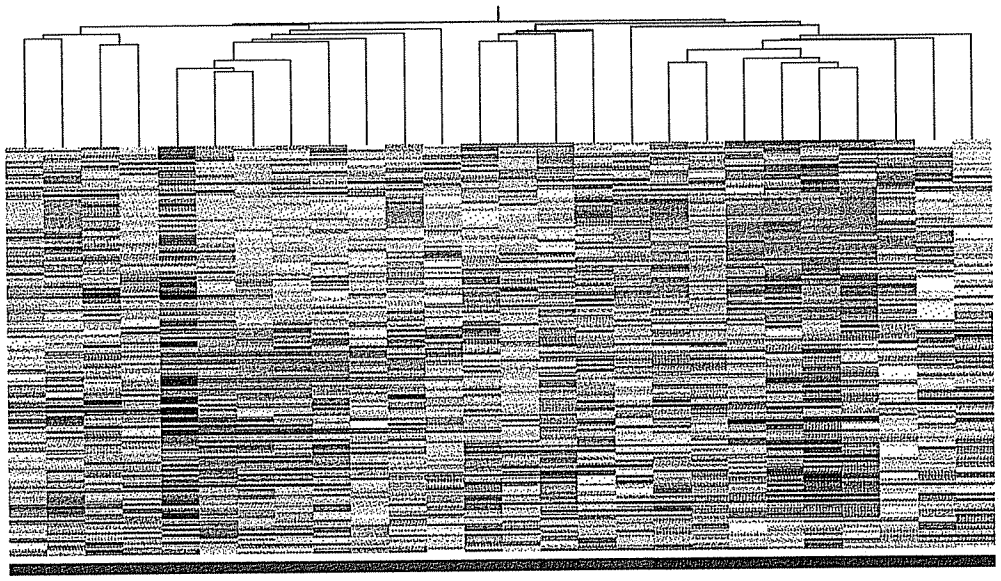
RA (n=10) Control (n=9)

**p<0.01**
**463 genes**

B. We have compared the 463 genes identified as noted above with the genes identified in each of the other lists described herein so as to demonstrate that the genes identified can be considered indicative of RA and not as part of a general and non specific disease response. We concluded that 302 of the genes identified are unique to RA and are not identified in the lists indicative of Colorectal Cancer, Osteoarthritis, Bladder Cancer, Heart Failure (HF), Coronary Artery Disease (CAD), Liver Cancer, Chagas Disease, Ovarian Cancer, Prostate Cancer, Alzheimer's, and Schizophrenia.

**Example 9 Bladder Cancer**

A. Microarray: a total of 26 samples (including 16 bladder cancer and 10 controls) were profiled using Affymetrix GeneChipU133 Plus2.0. A set of 157 probe sets, each corresponding to a gene ($p<0.005$, Wilcoxon-Mann-Whitney non-parametric test) were identified which individually demonstrate differential expression as between individuals having bladder cancer and individuals not having bladder cancer.

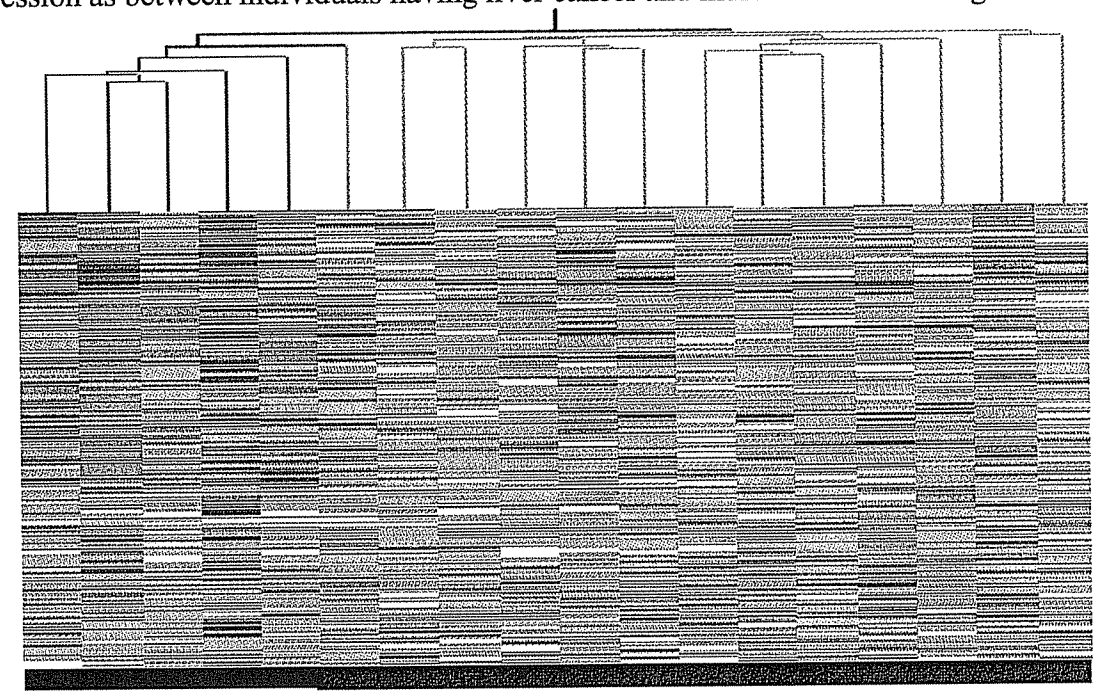



B. We have compared the 157 genes identified as noted above with the genes identified in each of the other lists described herein so as to demonstrate that many of the genes identified can be considered indicative of Bladder cancer and not as part of a general and non specific disease response. We concluded that 84 of the genes identified are unique to Bladder Cancer and are not identified in the lists indicative of Colorectal Cancer, Osteoarthritis, Alzheimer's, Heart Failure (HF), Coronary Artery Disease (CAD), Liver Cancer, Chagas Disease, Ovarian Cancer, Prostate Cancer and Rheumatoid Arthritis (RA).

**Example 10: Liver Cancer**

Microarray: a total of 18samples (including 5 liver cancer and 13 controls) were profiled using Affymetrix GeneChipU133A. A set of 457 probe sets, each corresponding to a gene ($p<0.05$, Wilcoxon-Mann-Whitney non-parametric test) which individually demonstrate differential

expression as between individuals having liver cancer and individuals not having liver cancer.

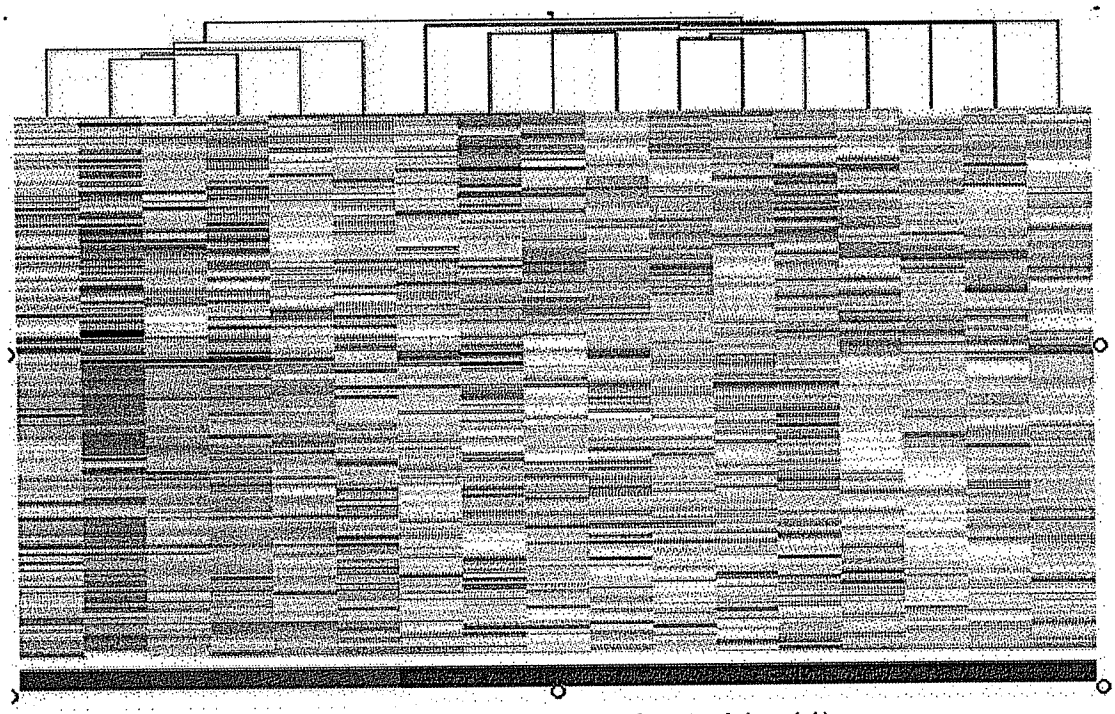



**p<0.05**
**457 genes**

B. We have compared the 457 probe sets identified as noted above with the probe sets identified in each of the other lists described herein so as to demonstrate that many of the genes identified can be considered indicative of liver cancer and not as part of a general and non specific disease response. We concluded that 209 of the probe sets identified are unique to Liver Cancer and are not identified in the lists indicative of Colorectal Cancer, Osteoarthritis, Alzheimer's, Heart Failure (HF), Coronary Artery Disease (CAD), Bladder Cancer, Chagas Disease, Ovarian Cancer, Prostate Cancer, Rheumatoid Arthritis (RA) and Schizophrenia.

**Example 11 Prostate cancer**

A. Microarray: A total of 17 samples (including 6 prostate cancer and 11 controls) were profiled using Affymetrix GeneChipU133 A. A set of 168 probe sets, each corresponding to a gene ($p<0.005$, Wilcoxon-Mann-Whitney non-parametric test) were identified which individually demonstrate differential expression as between individuals having prostate cancer and individuals not having prostate cancer.



Prostate cancer (n=6) Control (n=11)

**P<0.005**
**168 genes**

B. We have compared the 168 genes identified as noted above with the genes identified in each of the other lists described herein so as to demonstrate that many of the genes identified can be considered indicative of liver cancer and not as part of a general and non specific disease response. We concluded that 125 of the genes identified are unique to Prostate Cancer and are not identified in the lists indicative of Colorectal Cancer, Osteoarthritis, Alzheimer's, Heart Failure (HF), Coronary Artery Disease (CAD), Bladder Cancer, Chagas Disease, Ovarian Cancer, Liver Cancer, Rheumatoid Arthritis (RA) and Schizophrenia.

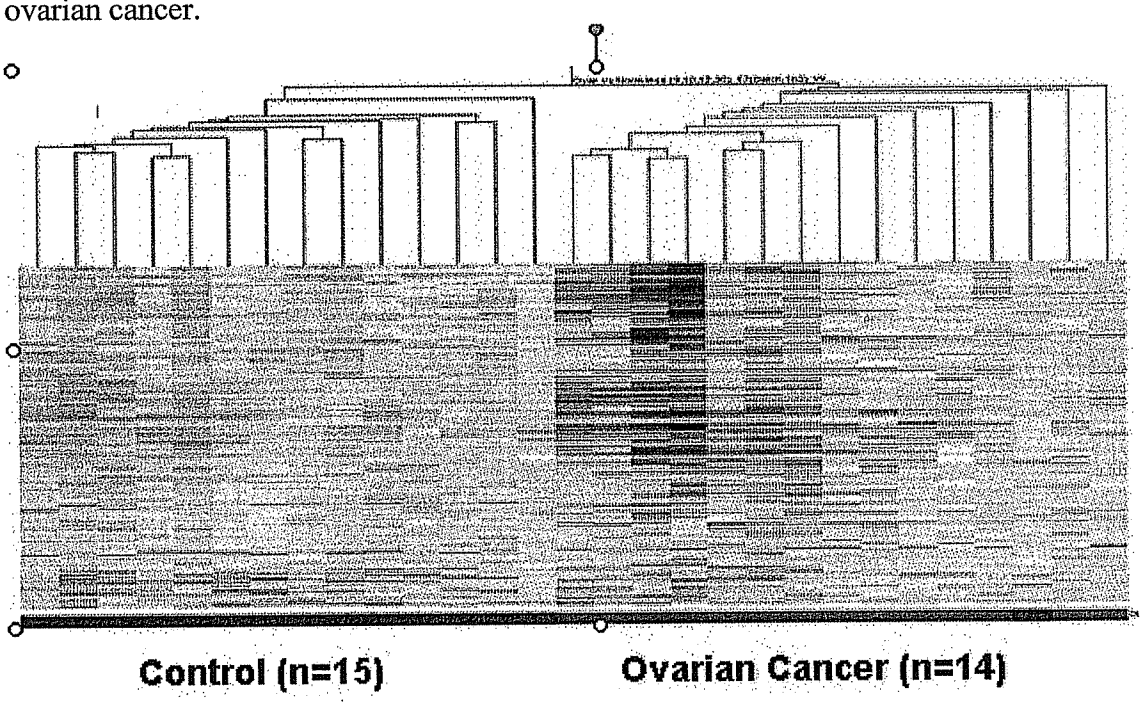
C. Real-time RT-PCR: The expression levels for 8 genes were confirmed as having statistically significant ($p<0.05$) differential expression as between control samples (n=30~31) and prostate cancer (n=34~37) samples.

**Prostate cancer vs. Ctrl**

| Affy ID | Sym | Affy result | | | | RT-PCR using more samples (30/34) | | |
|---|---|---|---|---|---|---|---|---|
| | | p value | PC/Ctrl | direction | sample size | p value | PC/Ctrl | direction |
| 212592_at | IGJ | 0.005 | 0.613 | ↓ | 11 Pca, 13 Ctrl | **0.006** | **0.547** | ↓ |
| 215813_s_at | PTGS1 | 0.008 | 0.754 | ↓ | 11 Pca, 13 Ctrl | **0.049** | 0.850 | ↓ |
| 209211_at | KLF5 | 0.008 | 1.397 | ↑ | 11 Pca, 13 Ctrl | **0.016** | 1.170 | ↑ |
| 209369_at | ANXA3 | 0.031 | 1.611 | ↑ | 11 Pca, 13 Ctrl | **0.034** | 1.305 | ↑ |

**Example 12: Ovarian Cancer**

A. Microarray: a total of 29samples (including 14 ovarian cancer and 15 controls) were profiled using Affymetrix GeneChipU133 A. A set of 277 probe sets, each corresponding to a gene ($p<0.001$, Welch t-test) were identified were identified which individually demonstrate differential expression as between individuals having ovarian cancer and individuals not having ovarian cancer.




**p<0.001**
**277 genes**

B. We have compared the 277 probe sets identified as noted above with the probe sets identified in each of the other lists described herein so as to demonstrate that many of the genes identified can be considered indicative of liver cancer and not as part of a general and non specific disease response. We concluded that 153 of the probe sets identified are unique to Ovarian Cancer and are not identified in the lists indicative of Colorectal Cancer, Osteoarthritis, Alzheimer's, Heart Failure (HF), Coronary Artery Disease (CAD), Bladder Cancer, Chagas Disease, Prostate Cancer, Liver Cancer and Rheumatoid Arthritis (RA).

C. Real-time RT-PCR: The expression levels for 7 genes were tested and 5 genes (three up-regulated genes, CEACAM1, ADIPOR1 and ADAM9; and two down-regulated genes, KIAA0562 and BCL11A) were verified as statistical significant ($p<0.05$) between control (n=15) and ovarian cancer (n=14).

I believe that this data presented in this declaration as an extension of the working examples already disclosed in the specification, provides further support for the enablement of the claimed methods of detecting a difference in expression at the RNA level of a gene which is expressed in blood and in a non-blood tissue in an unfractionated sample of whole blood from a human test subject versus a human control subject, wherein said difference is indicative of disease.

The data shows that for each of 11 diseases there is a statistically significant relationship between the disease phenotype and the differential expression of a gene identified as a marker gene, using a reasonable sample size. Further, the data shows that the majority of the differentially expressed genes are specific for the disease in question.

These claimed methods have been successfully applied to several other disease areas in data not shown here.

I further declare that all statements made herein of my own knowledge are true, and that all statements made on information and belief are believed to be true, and that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application and any patent issuing thereon.

C.C. Idew.....

Date: 28/02/06.....